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Genetic variation in the *CYP1A* gene caused by laboratory exposure of benzo (a) pyrene to common carp

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Polycyclic aromatic hydrocarbons (PAHs) are pervasive environmental contaminants, with benzo(a)pyrene (B(a)P) standing out as a prototypical example. B(a)P is a known mutagen and carcinogen that, upon metabolic activation stimulated by cytochromes P450 (CYP1A) with microsomal epoxide hydrolase, reacts with DNA. In this study, common carp (Cyprinus carpio) were subjected to three different doses of B(a)P (1µg/kg and 10µg/kg) to investigate the mutagenic effects on B(a)P-derived DNA in vivo.Blood samples were collected, followed by thorough DNA extraction and polymerase chain reaction analysis. The findings revealed a pronounced mutational impact of B(a)P on the amplified segment of the CYP1A gene. Specifically, at a concentration of 1µg/kg, B(a)P induced 8 mutations, 3 amino acid alterations, and the emergence of 5 new haplotype patterns. In contrast, at a concentration of 10µg/kg, B(a)P resulted in 21 mutations, 10 changes in amino acids, and the generation of 7 new haplotype patterns. Additionally, alterations in the three-dimensional protein composition were observed in both dosage groups.This study underscores the significant mutagenic potential of B(a)P, shedding light on its capacity to induce genetic changes and protein structure modifications, thereby emphasizing the importance of monitoring and addressing the environmental presence of PAHs for both ecological and human health considerations.

Keywords: Benzo(a)pyrene, *CYP1A* gene, mutations, common carp, Carcinogenicity, DNA extraction, Amino acid changes, Ecological health, DNA extraction.

INTRODUCTION

Nowadays, it is well known that the aquatic Ecosystem is more vulnerable to many sources of pollutants, so it can be considered a main reservoir for most pollutants that may accumulate in several parts of the aquatic organisms (Akhbarizadeh *et al.*, 2019; Zhao *et al.*, 2022). Fish occupy a major place in the food chain and have great commercial importance. In addition, from a human health perspective, contaminated fish is one of the most common pathways for persistent pollutants to which humans are exposed (Logan, 2007; Recabarren-Villalón *et al.*, 2021). PAHs are among the most influential organic pollutants in the aquatic environment (Ouro-Sama *et al.*, 2023). They are a class of long-lasting organic compounds that include two or more fused aromatic rings and are both lipophilic and hydrophobic. Pyrogenic, petrogenic, and biogenic processes are the principal producers of PAHs (Chen *et al.*, 2020a; Chen *et al.*, 2020b). PAHs are solid compounds that are either white, pale yellow, or colorless and are made up of fused carbon and hydrogen aromatic rings (Mallah *et al.*, 2022). Anthropogenic activities such as incomplete combustion of organic matter, petroleum contamination, terrestrial runoff, and atmospheric fallout from car exhaust and industrial emissions are the main sources of PAHs in the aquatic ecosystem (Ju *et al.*, 2022; Li *et al.*, 2018). One of the most cancer-causing priority PAHs and classified as a class I carcinogen by the International Agency for Research on Cancer is benzo(a)pyrene B(a)P (International Agency, 2021). Due to their procarcinogenic and mutagenic properties, many PAH compounds must undergo hepatic biotransformation in order to produce reactive metabolites and cause toxicity. These reactive metabolites, such as diolepoxides, which can covalently link to the DNA to create adducts, are produced during phase I transformation, namely by the cytochrome P450 monooxygenase system (Bramatti *et al.*, 2023; Chen, 2020). Stimulation of hepatic CYP1A in fish with certain classes of contaminants serves as an early warning system to determine levels of environmental contamination. This affects human intake of fish and the health of aquatic organisms (Andleeb *et al.*, 2022). Evaluating the possibility of causing mutations resulting from environmental exposures is very important to

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assess the risks they pose to the health of living organisms since cancer occurs due to mutations in bodily tissues (Hodgkinson and Eyre-Walker, 2011). The aim of the study is to detect the mutagenic susceptibility of B(a)P and the resulting genetic variation in the *CYP1A* gene of common carp.

MATERIALS AND METHODS

***Study sites*:** The study was carried out at Misan University's Animal House and Genetic Engineering Laboratory, which is part of the college of science's biology department**.**

***Test chemicals*:** B(a)P (Cas No.: 50-32-8, purity >96%) was obtained from Shanghai Macklin Biochemical (China). Dimethyl sulfoxide DMSO (purity >99%; Wako Pure Chemical Industries Ltd., Osaka, Japan) was used to dissolve the reagents in order to create test solutions.

***Test species*:** Thirty common carp fish at 10 individuals (average weight 35 gm/fish) per aquarium (60 cm × 30 cm × 30 cm) were randomly distributed in 3 glass aquaria filled with 46 l of dechlorinated tap water after treatment with a 1 ppm potassium permanganate solution for 90 minutes to get rid of external parasites (Protection, 1997).It was provided with 24-hour ventilation pumps, as well as temperature gauges, and filled with dechlorinated liquefied water, and its water was replaced by 50% per day with water kept in a large tank (capacity of 1 m3) equipped with an organized electric heater after leaving the water for 24 hours to ensure the removal of chlorine and maintain a temperature suitable for the life and growth of fish. The temperature was maintained at approximately 24 ± 1 degree using central air heating, and the amount of dissolved oxygen was maintained. At approximately 5 mg per liter and at a rate of lighting 12 hours a day, each of the was glass aquaria covered with a separate mesh cover to ensure that the fish remained in their designated and did not move to neighboring glass aquaria.

***Xenobiotic exposures*:** Dosing solutions consisted of B(a)P dissolved directly in DMSO. The first group (control) did not receive any of the test solutions. The second group was given a B(a)P solution with a concentration of 1 µg/kg. The third group was given a solution with a concentration of 10 µg/kg. The amount of dose was at 5 µL of test solution per gram of fish, according to Gerger and Weber (2015). It was given orally using Automatic Micropipettes rate of three doses for each fish, separating between one dose and the other for 48 hours. Then blood was drawn on the tenth day of dosing for a purpose Genomic DNA isolation and Polymerase Chain Reaction. All test fish had 0.5 ml of blood drawn from the caudal vein. These samples were gathered in EDTA tubes and stored at -18 °C in the freezer until the DNA is extracted.

Genomic DNA isolation and Polymerase Chain Reaction: DNA was extracted from whole blood using the Geneaid Company's (Taiwan) gSYNC TM DNA Extraction. Kit in accordance with the kit's protocol, and the DNA genome's presence was verified by electrophoresis on 1% agarose before the nanodrop instrument was used to calculate the samples' DNA concentration The range of 1.70 to 1.96 was found for the absorbance ratio of A260/28. Primer was selected for molecular detection and identification of mutations in the *CYP1A* gene, according to Kang *et al.* (2022). The primer was provided by the Korean company Macrogen, and its sequences are shown in (Table 1). PCR reactions were 25 µL; (Table 2) shows the reaction mixture and its amounts; the primer works; and the reaction takes place according to specific conditions shown in (Table 3).

Table 1. The sequence of CYP1A primer and length.

Gene	5`→ 3`)Sequences (		Size (bp)
CYP1A	exon 2	F: CTGAGCCTGACCGCTATGAG R: CCGCTTCCTACGATCTTCCC	503

Table 2. Materials used in PCR technology and their quantities.

Chemical	master mix	Genomic DNA	Primer		Sterile distilled water	total volume
			Reverse	Forward		
Volume (µl)	13	4	1	1	6	25

Table 3. Program for PCR-DNA Primers for CYP1A Gene.

PCR steps	Temperature (°C)	Time	Cycles
Initial denaturation	95	5 Min	1
Denaturation	95	30 Sec	35
Annealing	55	45 Sec	
Extension	72	45 Sec	
Final extension	72	10 Min	1
Final hold	4	5 Min	-

***Statistical analysis*:** Bioinformatics software (BioEdit, DnaSP 5.10, MEGA X, Network 5.0.0.0, and Phyre2 V.2.0) was used to detect mutation sites, determine total haplotypes (H), and draw the 3D protein.

RESULTS AND DISCUSSION

The results of the electrophoresis on the gel at a agarose concentration of 1% showed the success of the DNA amplification process, and the results of the electrophoresis on the gel at a agarose concentration of 1.5% showed the success of the PCR amplification process for the *CYP1A* gene If primer CYP1A gives a packet of size 503 bp in (Figure 1).

***Genetic Diversity*:** The results of the genetic diversity of the CYP1A gene for the group B(a)P 1µg/kg showed that the number of sites was 469 bp and the number of total haplotype

(H) were 5 haplotypes resulting in 8 genetic polymorphism (NH), and the values of haplotype diversity (HD) and nucleotide diversity (π) were 0.933 and 0.00848 respectively. The results of the genetic diversity of the B(a)P 10µg/kg group showed that the number of sites was 467 bp and the number of total haplotype (H) were 7 haplotypes resulting in 19 genetic polymorphism (NH), and the values of haplotype diversity (HD) and nucleotide diversity (π) were 0.900 and 0.01435 respectively (Table 4).

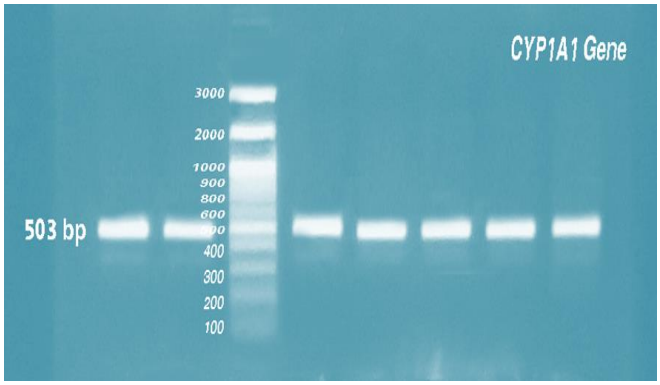

Figure 1. Electrophoresis of PCR product for the CYP1A gene (503 bp) using a 1.5% agarose gel with 70 V and 85 mA.

Table 4. Genetic Diversity of the CYP1A Gene among Fish Populations Treated with B(a)P at (1, 10 µg/kg) Concentrations

Compound	B(a)P1 µg/kg	B(a)P10 µg/kg
Number of Site (N)	469	467
Haplotype (H)	5	7
Number of polymorphic (NH)	8	21
Haplotype diversity (HD)	0.933	0.900
Nucleotide diversity (π)	0.00848	0.01435

***Single nucleotide polymorphisms*:** The results of the analysis of the nucleotide sequence (Sequencing) of the control group showed the absence of any change in the nitrogen bases.

***B(a)P1µg/kg group*:** In B(a)P1µg/kg group showed changes in the nitrogen bases in the axon 2, specifically at sites 519, 527, 528, 530, 546, 619, 795 and 838 of the CYP1A gene, as base G changed to C at sites (G519C), (G527C), C to T (C528T), G to A (G530A), C to A at sites (C546A), (C619A), A to T (A795T) and G to A (G838A) as shown in (Table 5) ، also showed that the formations G527C, G530A and G838A led to a change in genetic codes, which led to a change in amino acids at the level of protein produced from Serine (S) to Threonine (T), Serine (S) to Threonine (T), Arginine (R) to Lysine (K) and Glycine (G) to Serine (S) respectively, and the mutations were influential G519C, C546A, C619A and A795T, although their genetic codes changed from CTG to CTC, from GGC to GGA, from CGA to AGA and from ATA to ATT respectively. However, it gave the same amino acids, Leucine (L), Glycine (G), Arginine (R), and Isoleucine (I), respectively, meaning that the mutation is ineffective (silent) because these amino acids have more than one genetic code.

Diet is one of the most significant ways to be exposed to the widespread pollutant B(a)P in the aquatic ecosystem. Previous research has shown that it is present in many organisms' diets and tissues, as well as being biotransformed by lower organisms and then moving up the food chain (Jha, 2004; Wang and Wang, 2006). Studies have also demonstrated that this chemical is actively absorbed and metabolized when fish are consumed (Langan *et al.*, 2018; Peters *et al.*, 2002).

B(a)P is metabolized in the liver and converted into a number of metabolites after being ingested by organisms. Previous research has demonstrated that mutagenic substances produced by B(a)P metabolism, such as Reactive oxygen species (ROS) and B(a)P-7,8-dihydrodiol-9,10-epoxide (BPDE), because sDNA adducts and damage genomic integrity, which is crucial for cellular functions. Unrepaired

Table 5. Nucleotide changes and types of mutations, the resulting amino acid changes, and their impact on the translation process of the *CYP1A* gene to the group B(a)P1µg/kg.

Gene	Site[a] of SNP	Nucleotides (SNPs)	Triple code	Amino acids	Types of mutation	Effect of mutation on translation	Missense mutation %	Silent mutation %	Non-sense mutation %	Frameshift mutation %
CYP1A	519	G>C	CTG>CTC	L>L	Transversion	Silent	50	50	-	-
	527	G>C	AGC>ACC	S>T	Transversion	Missense				
	528	C>T	ACC>ACT	S>T	Transition	Missense				
	530	G>A	AGG>AAG	R>K	Transition	Missense				
	546	C>A	GGC>GGA	G>G	Transversion	Silent				
	619	C>A	CGA>AGA	R>R	Transversion	Silent				
	795	A>T	ATA>ATT	I>I	Transversion	Silent				
	838	G>A	GGT>AGT	G>S	Transition	Missense				

a: nucleotide sites for the *CYP1A* gene based on serial number XM_019064218.2 obtained from NCBI

SNP: single nucleotide polymorphism; C: Cytosine; T:Thymine; A: Adenine ;G : Guanine; L:leucine; S: Serine; T: Threonine; K: Lysine; R: Arginine; G: Glycine; I:Isoleucine; C: Cysteine.

DNA lesions also prevent transcription and replication, which can result in mutations and cancer (Alhmoud *et al.*, 2020; Cannan and Pederson, 2016; Papamichos-Chronakis and Peterson, 2013).

The results showed the mutational ability of B(a)P, which caused 8 mutations, which is consistent with a molecular study (Yuan *et al.*, 2013). on Gobiocypris rarus fish in which it was indicated that B(a)P caused increased expression of the *CYP1A* gene in liver, gills, and intestine tissue. the reason for this is due to the ability of the metabolites of the B(a)P compound to interact with DNA and form complexes that represent pre-mutation changes, which in most cases are identified and processed by repair systems, and in the event of obstacles that have not been removed, a point mutation begins in the form of replacement or deletion, where DNA complexes can induce mutations (Melendez-Colon *et al.*, 1999).Intermediate metabolic compounds resulting from the metabolism of B(a)P are associated covalently with DNA, leading to mutations, cytotoxicity cell transformation in vitro, and cancer induction in experimental animals. (Alexandrov and Thompson, 1977). Laboratory studies in which mice were treated with B(a)P orally or by diet showed an increase in the incidence of tumors in various organs such as the tongue, liver, lung, stomach, esophagus, lymphoid tissue, and hematopoietic tissues (Estensen *et al.*, 2004; Sparnins *et al.*, 1986; Agrawal *et al.*, 2018).

***B(a)P10µg/kg group*:** The results of the nucleotide sequence analysis (Sequencing) of the B(a)P10µg/kg group showed changes in the nitrogenous bases in the second axon, specifically at sites 463, 466, 485, 519, 523, 527, 528, 530, 540, 541, 546, 588, 599, 600, 611, 619, 709, 795, 804 and, 838 of the CYP1A gene as Rule G to A (G463A) and A to G changed at sites (A466G), (A485G), G to C (G519C), A to C (A523C), G to C (G527C), C to T (C528T), G to A (G530A), T to C (T540C), G to A (G541A), C to A(C546A), C to T (C588T), A to G sites (A599G), (A600G), A to - (A600-) and G to T (G611T) and C to A at sites (C619A), (C709A) and A to T (A795T), C to G (C804G) and G to A (G833A) and as shown in (Table 6). Table 6 also showed that the formations A466G, A485G, A523C, G527C, C528T, G530A, G541A, (A599G and A66G), G611T, and G833A led to a change in genetic codes, which led to a change in amino acids at the level of protein produced from Valine (V) to Methionine (M), Isoleucine (I) to Valine (V), Lysine (K) to Arginine (R), Serine (S) to Arginine (R), Serine (S) to Threonine (T) and Serine (S) to Threonine (T), Arginine (R) to Lysine (K), Aspartic acid (D) to Asparagine (N), Lysine (K) to Arginine (R), Serine (S) to Isoleucine (I) and Glycine (G) to Serine (S) on the tuly and mutations were influential, while G519C, T540C, C546A, C588T, C619A, C709A, A795T, and C804G, although their genetic codes changed from CTG to CTC, from GGT to GGC, from GGC to GGA, from CGC to CGT, from

Table 6. Nucleotide changes and types of mutations, the resulting amino acid changes, and their impact on the translation process of the *CYP1A* gene to the group B(a)P10µg/kg.

Gene	Site[a] of SNP	Nucleo-tides (SNPs)	Triple code	Amino acids	Types of mutation	Effect of mutation on translation	Missense mutation %	Silent mutation %	Non-sense mutation %	Frameshift mutation %
	463	G>A	GTG>ATG	V>M	Transition	Missense	57	38	-	5
CYP1A	466	A>G	ATC>GTC	I>V	Transition	Missense				
	485	A>G	AAA>AGA	K>R	Transition	Missense				
	519	G>C	CTG>CTC	L>L	Transversion	Silent				
	53	A>C	AGC>CGC	S>R	Transversion	Missense				
	527	G>C	AGC>ACC	S>T	Transversion	Missense				
	528	C>T	ACC>ACT	S>T	Transition	Missense				
	530	G>A	AGG>AAG	R>K	Transition	Missense				
	540	T>C	GGT>GGC	G>G	Transition	Silent				
	541	G>A	GAT>AAT	D>N	Transition	Missense				
	546	C>A	GGC>GGA	G>G	Transversion	Silent				
	588	C>T	CGC>CGT	R>R	Transition	Silent				
	599	A>G	AAA>AGA	---	Transition	Missense				
	600	A>G	AGA>AGG	K>R	Transition	Missense				
	600	A>-	---	---	Deletion	Frameshift				
	611	G>T	AGC>ATC	S>I	Transversion	Missense				
	619	C>A	CGA>AGA	R>R	Transversion	Silent				
	709	C>A	CCG>AGG	R>R	Transversion	Silent				
	795	A>T	ATA>ATT	I>I	Transversion	Silent				
	804	C>G	GGC>GGG	G>G	Transversion	Silent				
	83	G>A	GGT>AGT	G>S	Transition	Missense				

a: nucleotide sites for the *CYP1A* gene based on serial number XM_019064218.2 obtained from NCBI
SNP: single nucleotide polymorphism; C: Cytosine; T: Thymine; A: Adenine; G: Guanine; V: Valine; M: Methionine; I: Isoleucine; K: Lysine; R: Arginine; L: leucine; S: Serine; T: Threonine; G: Glycine; D: Aspartic acid; N: Asparagine.

CGA to AGA, from CCG to AGG, from ATA to ATT, and from GGC to GGG, respectively, It gave the same amino acids: Leucine (L), Glycine (G), Glycine (G), Arginine (R), Arginine (R), Arginine (R), Isoleucine (I), and Glycine (G), meaning that the mutation is ineffective (silent) due to the fact that these amino acids have more than one genetic code.

Some nucleotide sequences studied in exon 2 of the *CYP1A* gene were recorded for common carp at the National Center for Biotechnology Information (NCBI), the DNA Data Bank of Japan (DDBJ), and the European Nucleotide Archive (ENA) under the following accession numbers: LC768701 and LC768702.

The results showed a significant increase in the number of mutations by increasing the concentration from 1 to 10 (µg/kg), where the number of mutations increased from 8 to 21 mutations, and this is consistent with a laboratory study conducted by Gerger and Weber (2015). on adult zebrafish (*Danio rerio*) where the expression of CYPA1 increased by increasing the concentration of B(a)P by intraperitoneal injection (0.1,10,1000 µg/kg) or aqueous exposure (16.2,162 µg/l) for two days. It also agreed with a laboratory study Soltani *et al.*, (2019). on Liza klunzingeri fish where CYPA1 expression increased with an increase in the concentration of B(a)P by intraperitoneal injection (5,10,50 mg/kg), which indicated that this was associated with DNA damage. Similar effects have been observed by Kim and Hyun, (2006).when studying rainbow trout and common carp, which was also confirmed Costa *et al.*, (2011). When studying the effect of B(a)P on fish, it also agrees with our previous study on common carp (Hatem *et al.*, 2023).

***Three-Dimensional Protein Structure*:** The alignment of the amino acid sequence in the *CYP1A* gene for the groups of B(a)P1µg/kg, B(a)P10µg/kg with the joining number XM_019064218.2 (Figure2) revealed the presence of genetic mutations that led to the change of amino acids, as 3 changes were detected in the B(a)P1µg/kg group and 10 changes in the. B(a)P10µg/kg Bioinformatics software was used to predict three-dimensional protein structure to determine where amino acids change in the CYP1A protein in the aforementioned groups Figure 4, as these formations led to changes in the structure of the protein containing different functionally important regions, as shown in Figure 3.

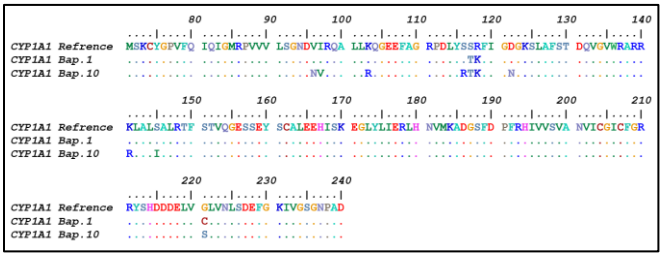

Figure 2. Comparison of amino acid sequence of the *CYP1A* gene groups B(a)P1µg/kg, B(a)P10µg/kg with accession number XM_019064218.2.

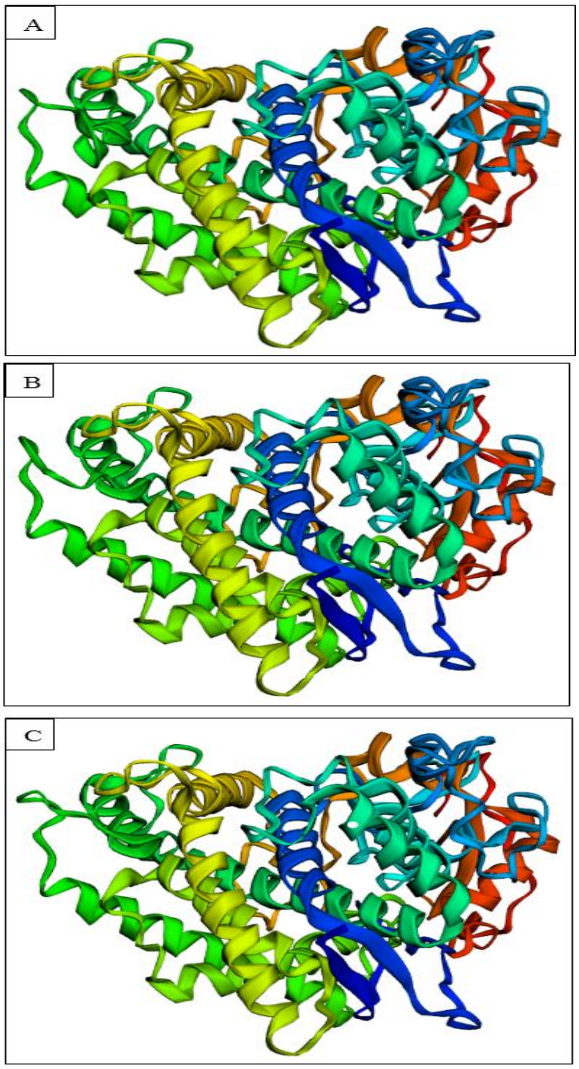

Figure 3. Three-dimensional forms of the CYP1A protein in (A) accession number XM_019064218.2, (B) B(a)P1µg /kg, (C) B(a)P1µg/kg.

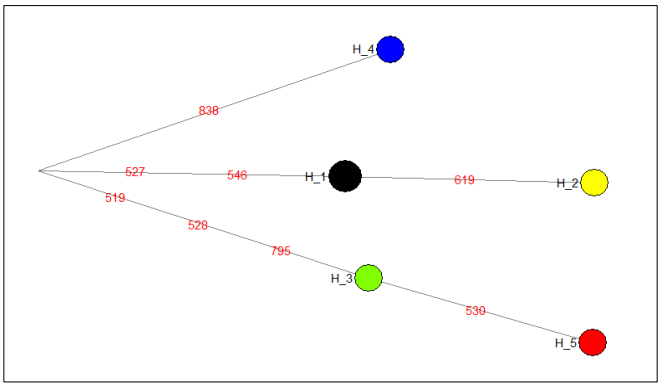

Figure 4. Haplotype Network of the *CYP1A* Gene for B(a)P1µg/kg Group.

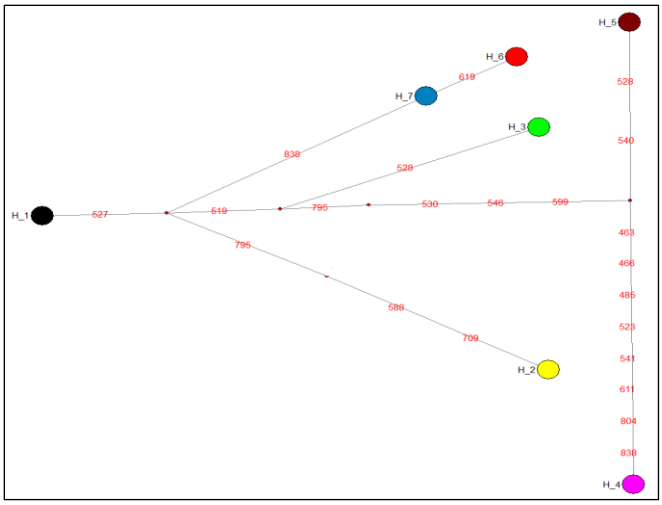

Figure 5. Haplotype Network of the *CYP1A* Gene for B(a)P10µg/kg Group.

***Conclusion*:** Our study showed that BAP has a high mutagenic potential, as it causes mutations and changes in the amino acids and three-dimensional protein structure in the *CYP1A* gene of common carp.

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***Authors' contributions*:** All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

***Conflict of Interest*:** We have no conflicts of interest to disclose.

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